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PRELIMINARY REPORT

Differences in Composition of Honey Samples and Their Impact on the Antimicrobial Activities against Drug Multiresistant Bacteria and Pathogenic Fungi

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Background an Aims. Antibiotic multiresistant microbes represent a challenging problem. Because honey has a potent antibacterial property, the antimicrobial effects of different honey samples against multiresistant pathogens and their compositions were investigated.

Methods. Five honey samples were used: Talah, Dhahian, Sumra-1, Sidr, and Sumra-2. Samples were analyzed to determine chemical composition such as fructose, glucose, sucrose, pH, total flavonoids, total phenolics, hydrogen peroxide concentration, minerals and trace elements. Antimicrobial activities of the samples against 17 (16 were multiresistant) human pathogenic bacteria and three types of fungi were studied. Specimens of the isolates were cultured into 10 mL of 10–100% (volume/volume) honey diluted in broth. Microbial growth was assessed on a solid plate media after 24 h and 72 h incubation.

Results. The composition of honey samples varied considerably. Sumra 1 and 2 contained the highest level of flavonoids and phenolics and the lowest level of hydrogen peroxide, whereas Dhahian honey contained the highest level of hydrogen peroxide. Sixteen pathogens were antibiotic multiresistant. A single dose of each honey sample inhibited all the pathogens tested after 24 h and 72 h incubation. The most sensitive pathogens were *Aspergillus nidulans*, *Salmonella typhimurum* and *Staphylococcus epidermidis* (*S. epidermidis).* Although there was no statistically significant difference in the effectiveness of honey samples, the most effective honey against bacteria was Talah and against fungi were Dhahian and Sumra-2.

Conclusions. Various honey samples collected from different geographical areas and plant origins showed almost similar antimicrobial activities against multiresistant pathogens despite considerable variation in their composition. Honey may represent an alternative candidate to be tested as part of management of drug multiresistant pathogens.

Key Words: Honey, Resistant, Bacteria, Fungi, Flavonoids, Hydrogen peroxide.

Introduction

The widespread use of antimicrobial agents has resulted in the development of antibiotic resistance, which has a serious impact on the general community. New compounds are being developed to overcome the challenge of antimicrobial resistance. However, a rapid development of resistance to some of these newer agents is reported. Honey has been long used to treat infected leg ulcer, earache, measles, eye diseases, and gastric ulcers (1,2). Honey was mentioned in the Talmud, both the old and new testaments of the Bible, and the Holy Quran. In the Surat AL-Nahel (The Bee) it

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says: And thy LORD taught the bee to build its cells in hills, on tree and in men's habitations, then to eat of all the produce of the earth and find with skill the spacious paths of its LORD, there issues from within their bodies a drink of varying colors, wherein is healing for men, verily in this is a sign for those who give thought. The Muslim prophet Mohammad recommended the use of honey for the treatment of diarrhea. There is increased interest in using honey as an antibacterial agent as well as a wound dressing (3,4).

Different honeys vary in the potency of their antibacterial activity, which may be due to variations in plant source (5,6). Much of the literature on the use of honey in microbial infections and wound healing does not give the type of honey used. Honeys collected from different geographical areas showed various activities (7,8). All honey is not equal in its effectiveness (9,10). However, few clinical reports disclosed the specific type of honey applied to infected wounds and ulcers. A wide range of MIC of honey has been reported in studies comparing different types of honey tested against single species of bacteria: from 25−0.25% (v/v); 50−1.5% (v/v); 20−0.6% (v/v); 50 to 1.5% (v/v) (11,12).

Honey solutions collected from both the U.S. and New Zealand inhibited 24/28 *Helicobacter pylori* isolates at a concentration of 10% and 28/28 isolates at a concentration of 15% (13). In the UK, *Burkholderi acepacias s*trains were susceptibility to concentrations of honey <6% (v/v) (14). A study from Iran showed that growth of mycobacteria is inhibited by adding 10% honey to the media (15). A study conducted by the authors to evaluate the activity of Emirates honey toward human pathogens showed that honey (30−70%) prevents growth of Gram-positive and -negative bacteria and *C. albicans* (16). MIC of 11 samples of stingless bee honey collected in Australia ranges from 4−10% for Gram-positive bacteria, 6−16% (w/v) for Gram-negative bacteria and 6−10% (w/v) for *Candida spp* (17). MICs of Malaysian tualang honey toward bacteria ranged from 8.75-25% compared to manuka honey (8.75−20%) (18). The MIC of the Algerian honeys ranged from 12−18% (v/v) when tested against *P. aeruginosa* (19).

In the United Arab Emirates (UAE), we found that multifloral honey completely inhibits the growth of Gram-negative and -positive isolates at concentrations of 30% and higher (16). In The Netherlands, honey kills *B. subtillis,* methicillin-resistant *S. aureus*, extended-spectrum beta-lactamase producing *E. coli*, ciprofloxacin-resistant *P. aeruginosa*, and vancomycin-resistant *Enterococcus faecium* at concentrations of 10−20% (volume/volume) (20). After 2 days of topically applied honey, the extent of forearm skin colonization in healthy volunteers was reduced 100-fold, and the numbers of positive skin cultures were reduced by 76% (21). A pasture honey and a manuka honey had bactericidal activity when diluted >10-fold against 17 strains of *P. aeruginosa* (22). We demonstrated that 80% honey concentration inhibited growth of small (1 µl) and large size of inoculums (10 µl) of *E. coli* and *S. aureus* when added to their cultures within 24 h inoculation. It was concluded that the therapeutic period of honey and recovery of the inhibited isolates requires that the dose of honey is adjusted according to type of isolate and rate of growth (23).

Lactic acid bacteria isolated from honey had antibacterial activities against multiple antibiotic resistant *S. aureus, S. epidermis* and *Bacillus subtilis* (24). More than 2000 bacterial strains isolated from six U.S. domestic honeys and two manuka honeys from New Zealand showed a high incidence of antimicrobial inhibition. The high rate of antimicrobial activity exhibited by these bacterial strains could provide potential sources of novel antimicrobial compounds (25).

In the Arabian peninsula there are many kinds of honeys produced in various areas where people are using honey for management of many diseases. In this regard, samples of five different honeys were collected, and their antibacterial actions against standard and recently isolated human pathogens that exhibit multiresistance to various antibiotics were studied. Some of their physical and chemical compositions were determined.

Materials and Methods

Pathogenic Isolates

Cultures of various human pathogenic strains were obtained from the Microbiology Department, College of Pharmacy, King Saud University, Riyadh. The species included *E. coli, Staphylococcus aureus, B. subtillis, P. aeruginosa, Klebsiella pneumonia, Salmonella typhimurum, Micrococcus luteus, S. epidermidis, Bacillus cereus, Aspergillus nidulans, Serratia marcescens,* and *Enterobacter aerogene.* These strains were isolated from human specimens. The isolates were identified by the standard bacteriological techniques. Standard isolates included *Candida albicans* ATCC 10231, *C. tropicalis* ATCC 10231, *E. coli* ATCC 10402, *Salmonella typhimurum* ATCC 3311, *B. subtillis* ATCC 10402, *S. aureus* 29212, *P. aeruginosa* ATCC 2785, and *Klebsiella pneumonia* ATCC 10031. These were used to demonstrate if there is any difference in the antibacterial activity of honey against recently isolated human pathogens from standard strains. The Kirby-Bauer method was used to test antibiotic sensitivity. Using a 10-µl standard loop, a colony of each isolate was picked from a plate, grown in 10 mL nutrient broth, and used after 24 h culture in 37°C for bacteria and at 30°C for fungi. The cultural media and materials were ready made and supplied by King Saud University.

Honey

Five different honey samples were collected (Table 1). All samples showed no signs of granulation or fermentation.

Table 1. Types and origin of honey samples used in the experimentation

Honey color designations	Area of collection	Plant origin	Name of honey
Amber	Al-Bahah	Acacia Johnwoodii	Talah
Extra light amber	Al-Bahah	Acacia ask Forissk. Willd	Dhahian
Dark amber	Tahama	Acacai Tortilis-1	Sumra-1
Light amber	Tahama	Zizphusspina-christi	Sidr
Dark amber	Tahama	Acacai Tortilis-2	Sumra-2

Samples were analyzed to determine chemical composition such as fructose, glucose, sucrose, pH, total flavonoids, total phenolics, hydrogen peroxide concentration, minerals and trace elements. The volume of honey necessary to achieve the required concentrations (10−100%, volume/volume) was aseptically added into sterile test tubes and then nutrient broth was added to obtain the required honey concentration. Honey broth solutions were mixed by stirring with vortex. Macrodilution was used to measure minimum inhibitory concentration (MIC) of honey.

Honey Analysis

Total phenolic content. Folin−Ciocalteu method was used to assay total phenolic content, which was described by Moreira et al. (26). Honey sample was diluted in MeOH (500 µl of 1:10 g mL^{-1}) and was mixed with 500 µL of the Folin−Ciocalteu reagent and 500 µl of Na_2CO_3 (10% w/v). The absorbance of the reaction mixture at 700 nm was determined against the blank after incubation for 1 h. Blank was prepared as above without the honey. Gallic acid standard solutions (0.01−0.08 mM) were used for the calibration curve (y = 2.3727x + 0.0022; R2 = 0.9998) and the total phenol content was expressed as mg of gallic acid equivalent/306/kg of honey.

Total flavonoid content. For flavonoid contents, the aluminum chloride method was used (27). Honey (250 µl) was mixed with 1.25 mL of distilled water and 75 µl of a 5% $NaNO_2$ solution. A total of 150 µL of a 10% aluminum chloride-water solution was added after 5 min. Six minutes later, 500 µl of 1M NaOH and 275 µl of distilled water were added to the mixture and vortexed. The intensity of the pink color of the reaction mixture at 510 nm is determined against the blank, which was prepared as above without the honey. Catechin standard solutions (0.022−0.34 mM) were used for the calibration curve (y = 315 0.9652x−0.0091; R2 = 0.9981). Total flavonoid content was expressed as mg of catechin equivalents 316/kg of honey.

Hydrogen peroxides. Hydrogen peroxide/peroxidase assay kit was used for hydrogen peroxide analysis (Amplex Red, Molecular Probes, Invitrogen, Burlington, ON, Canada) (28). The assay was conducted in the 96-well microplates according to the manufacturer's instruction. The fluorescence of the formed product, resorufin, was measured at 530 nm excitation and a 590 nm emission using the Synergy HT (Molecular Devices, BioTek Instruments, Winooski, VT) multidetection microplate reader, and the dose−response curves were generated using the KC4 data reduction software. To calculate the hydrogen peroxide concentrations of the honeys, a standard curve was run alongside the honey serial dilutions. The standard curve was prepared from the 200 µM H_2O_2 stock solution. Each of the honey samples and the standard curve were tested in triplicate.

Sugar Analysis by HPLC

Sample collection. Honey samples were stored at 4°C until analysis. The AOAC method was used to extract sugars from the honey samples (29). Each honey sample (5 g) was weighed in a small beaker, transferred to a 50-mL volume flask with 25 mL water. It was immediately diluted to volume with acetonitrile. Final solution was passed through a C18 Chroma bond cartridge (Macherey Nagel) and the eluant was filtered through 0.45-µm filter. The standard solution was prepared daily by placing 3.82 g fructose, 3.00 g glucose, 0.26 g sucrose, into 100 mL volume flask, dissolved in 50 mL of water, adding acetonitrile to volume.

Sugar Analysis by HLBC

The HPLC method of AOAC was followed except that acetonitrile-water mobile phase and solvent flow rate used were 80/20 and 1.5 mL/min, respectively, to improve the separation of the individual sugars on the column. Thus, separation of compounds was achieved with an analytical HPLC unit (Perkin Elmer, Waltham, MA), using a refractive index detector and Amino Column. A 20-µl portion of each prepared honey sample was injected and the sugar content was calculated by external standard method using peak areas. A 20-µl aliquot of the mixed sugar standard was injected in duplicate at the beginning, during, and at the end of each day's run to check retention time and peak area.

Mineral Analysis

The analysis was performed on a multi-element Inductively Coupled Plasma Mass Spectrometer (ICP-MS), Model ELAN6100, Perkin Elmer (PE), Sciex Instruments (Waltham, MA) equipped with standard torch, cross flow nebulizer, Ni sampler and skimmer cones (30). A 0.25 g honey sample was weighed and transferred to a teflon digestion tube (120 mL). Then, 5 ml of HNO_3 + 2 mL H_2O_2 +1 mL HF was added. The tube was sealed and the sample was digested using a microwave oven (Milestone ETHOS 1600). After digestion is completed, samples were cooled to room temperature. Deionized water was then added

and the mixture was heated on a hot plate (120°C) for 30 min to remove the HF. The resulting digest was filtered in a graduated plastic tube using 1% HNO_3 and the volume was brought to 50 mL. Later, 1 mL of solution was diluted to 10 mL using 1% HNO_3. Samples were prepared in a batch of six including a blank (HNO_3/H_2O_2/HF).

Effects of Honey on Human Pathogens

Experiment 1. In order to study the antimicrobial activity of the selected honeys on the common human pathogenic isolates and to measure MIC with use of broth macrodilution method, a specimen of each microorganism was taken from pure culture grown in the 10-mL nutrient broth as described above. These specimens were cultured in broth containing different concentrations of honey (10–100% volume/volume) by using a standard loop (10 µl) to measure MIC. For each type of bacteria culture, 10 µl of 3.6×10^7 CFU/ml bacteria suspension was inoculated into 10 mL honey/broth medium and incubated at 37°C for 24 h. For fungal pathogen culture, 10 µl of 5 x 10^8 CFU/ml fungal suspension was inoculated into 10 mL honey/broth medium and incubated in 30°C for 24 h. Afterwards, a loopful (10 µl) of the culture of each specimen of the microorganism was streaked onto agar plates. The streaked plates were incubated aerobically at 37°C for bacteria and at 30°C for fungi and inspected after 24 h. Microbial growth was assessed visually on solid media as follows: 0 colonies = no growth, 1–5 colonies = little growth, 6–20 colonies = mild growth, 21–50 colonies = moderate growth, >50 and uncounted colonies = heavy + growth and uncounted colonies + full streak growth = very heavy growth. The experiment was performed in duplicate for each culture to verify the results.

Experiment 2. In order to study whether a single dose of each honey is capable of inhibiting the growth of the isolates after 3 days, cultures of the 20 pathogens in the appropriate control liquid broth media and in the 10–100% honey concentration were incubated for 72 h at 37°C. After 72 h incubation, a loopful specimen of each isolate cultured in liquid broth or 10–100% honey concentration was streaked onto solid media prepared in petri dishes to assess growth of the cultured bacteria and fungi as described in experiment 2. The experiment was performed in duplicate for each culture to verify the results.

Statistical Analysis

Mean and standard deviation of MIC of the honeys against Gram-positive and Gram-negative bacteria and fungi were measured. ANOVA and *t* test were used to compare means of MIC of the honey samples; $p < 0.05$ was statistically significant.

Table 2. TDS, moisture and mineral composition of the different five honey samples

Variables	Types of honey					Highest level
	Talah	Dhahian	Sumra-1	Sidr	Sumra-2	
TDS	84.5	83.3	84.9	84.7	84.9	Sumra 1
Moisture	15.5	16.7	15.1	15.3	15.1	Dhahian
Na	487	472	488	494	517	Sumra 2
Mg	20.3	2.59	2.13	4.89	23.7	Sumra 2
Al	4.01	3.85	3.6	3.65	4.6	Sumra 2
K	478	41.6	499	232	629	Sumra 2
Ca	15.6	11.1	16.2	10.4	26.1	Sumra 2
Cr	0.055	0.066	0.061	0.062	0.100	Sumra 2
Mn	0.115	0.031	0.107	0.041	0.169	Sumra 2
Co	0.0030	0.0017	0.0041	0.002	0.0075	Sumra 2
Ni	0.0387	0.0245	0.0318	0.0341	0.0662	Sumra 2
Cu	0.025	0.043	0.172	0.037	0.214	Sumra 2
Zn	0.326	0.131	0.283	0.186	0.335	Sumra 2
Rb	0.251	0.011	0.116	0.135	0.134	Talah
Sr	0.060	0.054	0.095	0.070	0.118	Sumra 2
Ba	0.025	0.019	0.016	0.014	0.022	Sumra 2
Pb	0.0037	0.0075	0.0043	0.0042	0.0076	Sumra 2

Note: Units expressed in mg/100 g of honey.

Results

Composition of honeys is shown in Table 2. Darker honey (Sumra-2) contains relatively higher levels of minerals such as Cu, Mg, K, Ca, Mn, K, and Zn. Sidr honey has the highest pH and lowest total phenolics and flavonoid contents and sugars among the honey samples (Table 3). Talah honey had high flavonoids and phenolic contents compared to Dhahian and Sidr. Sumra 1 and Sumra 2 had the highest flavonoid and phenolic content and lowest concentration of hydrogen peroxide. Dhahian honey has the highest concentration of hydrogen peroxide, lowest pH, and low flavonoid and phenolic contents. There is an inverse relationship between flavonoid and phenolic contents and hydrogen peroxide concentration. Darker honey, Sumra 1 and Sumra 2 contain the highest content of flavonoids and phenolics.

The antimicrobial sensitivity test demonstrated that 16/17 of the bacterial isolates showed antibiotic multiresistance (Table 4). All the honey samples inhibited gram-positive and -negative pathogens and fungi (standard strains and newly isolated human pathogens) (Table 5). There was no statistically significant difference between antimicrobial activities among the honey samples except for Talah honey, which had significantly lower activity against fungi (Table 5). None of the isolates showed resistance to any of the honey samples. Upon comparison among the antimicrobial activities of the five honey samples, no statistically significant difference was found between their MIC against the 20 isolates. The mean ± SD of MIC for the honey samples against all the microbes were Talah honey (46.0 ± 13.53), Dhahian honey (42.0 ± 11.05), Sumra-1 honey (44.0 ± 11.42), Sidr honey (41.5 ± 9.33), and Sumra-2 honey (40.5 ± 7.59), F = 0.83.

Table 3. Differences in the acidity, total flavonoids, phenolic contents, and sugar composition of the five honey samples

Variables	Honey samples					F/*p* values
	Talah	Dhahian	Sumra 1	Sidr	Sumra 2	
Total phenolics (mg/GAE/ 100 g honey)	63.2 ± 1.98	53.5 ± .70	72.8 ± 1.6	23.90 ± 0.60	68.3 ± 1.3	652/0.00
Total flavonoids (mg/100 g honey)	28.2 ± 1.2	19.3 ± 1.3	44.1 ± 1.8	13.4 ± 0.40	32.3 ± 2.1	197/0.00
Hydrogen peroxide concentration (mM/l)	0.71 ± .02	1.11 ± .04	0.38 ± .02	1.04 ± 0.18	0.34 ± 0.03	54/0.00
Glucose g%	21.2 ± 1.2	20.84 ± 0.7	21.18 ± 0.9	20.75 ± 1.1	27.80 ± 1.8	19.4/0.0001
Fructose g%	34.96 ± 1.8	38.24 ± 2.1	36.42 ± 1.3	30.38 ± 1.9	31.32 ± 1.8	10.3/0.001
Sucrose g%	2.73 ± 0.2	2.02 ± 0.3	2.78 ± 0.4	2.54 ± 0.6	2.75 ± 0.2	2.2/0.14
pH	3.70 ± 01	3.45 ± 01	4.80 ± 05	6.14 ± 03	4.70 ± 04	33.5/0.00

Fungi were less susceptible to all honey samples than bacteria. MICs of the honey samples against Gram-positive and -negative bacteria were lower than that for fungi, particularly for Talah honey. The susceptibility of each fungus to honey samples was different from other fungi (Table 5).

The most susceptible isolates to honey samples among Gram-negative bacteria were *Salmonella typhimurium* (mean MIC 34 ± 5.4), *Salmonella typhimurium* ATCC 3311 (mean MIC 36 ± 5.4), and *P. aeruginosa* ATCC2785 (mean MIC 38 ± 8.3) (Table 5). Regarding Gram-positive bacteria, *S. epidermidis* (mean MIC 38 ± 10.9) and *B. subtillis* (mean MICs 38 ± 8.3) were more susceptible to honey sample treatment. *Salmonella typhimurium, S.aureus, S. epidermidis* and *Aspergillus nidulans* were most susceptible to the lowest MIC of most of the honey samples (Table 5). Isolates had different susceptibilities to the honey samples. Table 6 showed the best honey with lower MIC (30% and less) for each of te pathogens.

Small doses of Talah honey (MIC 30% or less) inhibited a higher number of bacterial isolates than other honey samples, particularly after 72 h. Therefore, it has a higher potency against most of the bacterial isolates when compared to other samples. It has less effect against fungi than the other honey samples. Therefore, Talah honey shows more effectiveness against bacteria and less effectiveness against fungi. Sidr honey has the lowest potency against most of the isolates although its antimicrobial effect against entire isolates did not significantly differ from the other samples. Sumra-2, Sumra-1 and Dhahian had almost similar potency against the isolates.

The grade of growth of the isolates in the control and honey containing media were similar when tested after 24 h and 72 h culture; both showed very heavy growth. In addition, a single dose of each honey sample inhibited the isolated pathogens when their growth was tested after 24 h and 72 h incubation (Table 5). This demonstrated that a single dose of each honey tested (MIC) was capable of inhibiting the growth of the isolates for 72 h. When cultures of the microbes in control liquid media and in media containing various concentrations of the honey samples incubated for 72 h, most of the isolates became more susceptible to one or more honey sample, particularly *E. coli, S. aureus, S. epidermidis, P. aeruginosa,* and all fungi (Table 6). The mean MIC of honey samples was significantly lower after 72 h against *C. tropicalis* and *Enterobacter aeruginosa* ($p < 0.05$). This means that some of the isolates showed a lower grade of growth in media containing honey after 72 h culture when compared to their growth after 24 h culture.

Table 4. Drugs to which the isolates are resistant

Name of microbes	Name of drugs
E. coli ATCC 10402	LZG, VA 30, E 15, CXM, AMC 30
E. coli[a]	LZG, VA 30, E 15, CXM, AMP 25
Bacillus subtilis ATCC 10402	CXM, AMC 30, FOX 30, AMP 25
Bacillus subtilis[a]	CXM, AMC 30, FOX 30, AMP 25
S. aureus 29212	CXM, AMC 30, AMP 25
S. aureus[a]	C 30, CXM
P. aeruginosa ATCC 2785	F 300, LZG, C 30, VA 30, E 15, CXM
P. aeruginosa[a]	K 30
Klebsiella pneumonia ATCC 10031	VA 30, CXM, AMP 25
Klebsiella pneumonia[a]	LZG, VA 30, AMP 25
Salmonella typhimurum ATCC 3311	LZG, VA 30, E 15, CXM, AMC 30, FOX 30
Salmonella typhimurum[a]	LZG, VA 30, E 15, CXM,
Micrococcus luteus[a]	F 300, CXM, AMC 30, FOX 30, AMP 25
S. epidermidis[a]	-
Bacillus cereus[a]	CXM
Enterobacter aerogenes[a]	LZG, CXM
Serratia marcescens[a]	LZG, VA 30, E 15
C. albicans ATCC 10231	-
C. tropicalis ATCC 10231	-
Aspergillus nidulans[a]	-

F 300, nitrofurantoin; LZD, linezolid; C 30, chloramphenicol, VA 30, vancomycin; E 15, erythromycin; CIP, ciprofloxacin; CXM, cefuroxime; K30, kanamycin; AMC30, amoxicillin; FOX30, cefoxitin; AMP25, ampicillin.
[a]Clinical isolates.

Discussion

The results demonstrated that all five types of honey are effective against all the drug multiresistant standard and recently isolated human pathogens. None of the microbes showed resistance to any of the honey samples. The most sensitive microbes are *Aspergillus nidulans* as compared

Table 5. MIC of honey (Talah, Dhahian, Sumra -1, Sidr and Sumra -2) against Gram-positive and -negative bacteria and fungi after 24 and 72 h incubation, Clinical isolates, VH, very heavy growth

			MIC (% volume/volume) of various types of honey				
Bacteria	Time of culture (h)	Control	Talah	Dhahian	Sumra-1	Cidar	Sumra-2
E. coli ATCC 10402	24	VH	40%	40%	40%	40%	40%
	72	VH	30%	40%	40%	40%	30%
E. coli	24	VH	50%	40%	40%	40%	40%
	72	VH	40%	40%	40%	40%	40%
P. aeruginosa ATCC 2785	24	VH	30%	30%	50%	40%	40%
	72	VH	30%	40%	40%	40%	40%
P. aeruginosa	24	VH	50%	40%	60%	30%	40%
	72	VH	30%	40%	50%	40%	40%
Klebsiella pneumonia ATCC 10031	24	VH	40%	50%	40%	40%	30%
	72	VH	40%	40%	60%	40%	20%
Klebsiella pneumonia	24	VH	40%	40%	40%	40%	40%
	72	VH	50%	50%	30%	40%	40%
Salmonella typhimurum ATCC 3311	24	VH	30%	30%	40%	40%	40%
	72	VH	30%	40%	40%	40%	40%
Salmonella typhimurum[a]	24	VH	30%	30%	30%	40%	40%
	72	VH	30%	30%	30%	30%	40%
Serratia marcescens[a]	24	VH	30%	50%	40%	40%	40%
	72	VH	50%	70%	40%	40%	40%
Enterobacter aerogenes[a]	24	VH	50%	40%	40%	50%	40%
	72	VH	40%	40%	30%	40%	40%
S. aureus 29212	24	VH	40%	40%	40%	50%	50%
	72	VH	30%	50%	40%	50%	50%
S. aureus	24	VH	40%	30%	40%	50%	40%
	72	VH	40%	30%	30%	20%	40%
Bacillus subtilis ATCC 10402	24	VH	60%	70%	50%	30%	30%
	72	VH	70%	40%	50%	40%	30%
Bacillus subtilis	24	VH	40%	30%	40%	50%	30%
	72	VH	40%	40%	40%	40%	30%
S. epidermidis[a]	24	VH	20%	50%	40%	40%	40%
	72	VH	10%	50%	30%	10%	40%
Micrococcus luteus[a]	24	VH	40%	40%	30%	40%	50%
	72	VH	50%	30%	30%	40%	50%
Bacillus cereus	24	VH	40%	50%	40%	40%	40%
	72	VH	40%	70%	40%	30%	40%
C. albicans ATCC 10231	24	VH	70%	40%	70%	60%	50%
	72	VH	40%	50%	50%	60%	50%
C. tropicalis ATCC 10231	24	VH	60%	50%	40%	60%	60%
	72	VH	40%	50%	40%	40%	50%
Aspergillus nidulans	24	VH	50%	50%	40%	30%	30%
	72	VH	50%	30%	40%	40%	30%

VH, very heavy growth; MIC, minimum inhibitory concentration.
[a]Clinical isolates.

to other fungi; *Salmonella typhimurum*, *P. aeruginosa* and *E. coli* as compared to other Gram-negative isolates; and *S. epidermidis* and *B. subtilis* as compared to other Gram-positive isolates. Although there was no statistically significant difference in the effectiveness of all honey samples, the most effective honey against fungi were Sumra-1 honey and Sumra-2 honey and against Gram-negative isolates was Sumra-2 honey. In the Gram-positive group, the five types of honey have nearly similar activities. In a previous study, we found that the most sensitive microbes to Emirates honey were *E. coli, P. aeruginosa,* and *Haemophilus influenzae* (16).

After 72 h culture in the appropriate media, the grade of growth of all isolates was similar to the grade of their growth when cultured for 24 h. However, when the isolates were incubated in various concentrations of the honey samples, some of the isolates showed a lower grade of growth after 72-h culture as compared to their growth after 24-h culture. Talah honey and Sumra 2 honey became more effective after 72 h. This means that either the isolates gained more susceptibility to the inhibition of the honey with increasing time of exposure or the single dose of honey cause irreversible damage in the isolates, leading to their death when cultured longer than 24 h. This theory needs further testing.

Table 6. Types of honey with lowest MIC against each isolate and the difference in susceptibility of each isolate to all honey samples between 24-h and 72-h cultures

Pathogenic isolates	Type of honey with lowest MIC against each isolate		Mean ± SD MIC of the five honey samples		
	After 24 h	After 72 h	After 24 h	After 72 h	*p* value
C. albicans ATCC 10231	Dhahian	Talah	58 ± 13	50 ± 7.0	0.088
C. tropicalis ATCC 10231	Sumra-1		54 ± 8.9	44 ± 5.9	0.01
Aspergillus nidulans	Sidr, Sumra-2	Dhahian, Sumra-2	40 ± 10	38 ± 8.3	0.36
E. coli ATCC 10402		Talah, Sumra-2	40 ± 0.0	36 ± 5.6	0.07
E. coli			42 ± 4.7	40 ± 0.0	0.18
Bacillus subtilis ATCC 10402	Sidr, Sumra-2	Sumra-2	38 ± 8.3	38 ± 4.4	0.49
Bacillus subtilis	Dhahian, Sumra-2	Sumra-2	38 ± 8.3	38 ± 4.4	0.49
S. aureus 29212		Talah	44 ± 5.4	44 ± 8.9	0.49
S. aureus	Dhahian	Sidr, Dhahian, Sumra-1	40 ± 7.0	32 ± 8.3	0.06
P. aeruginosa ATCC 2785	Talah, Dhahian	Talah	38 ± 8.3	38 ± 4.4	0.49
P. aeruginosa	Sidr	Talah	44 ± 11	40 ± 7.0	0.25
Klebsiella pneumonia ATCC 10031	Sumra-2	Sumra-2	40 ± 7.0	40 ± 14	0.49
Klebsiella pneumonia		Sumra-1	40 ± 0.0	42 ± 8.3	0.30
Salmonella typhimurum ATCC 3311	Dhahian, Talah	Talah	36 ± 5.4	38 ± 4.4	0.26
Salmonella typhimurum	Talah, Dhahian, Sumra-1	Talah, Dhahian, Sumra-1, Sidr	34 ± 5.4	32 ± 4.4	0.26
Micrococcus luteus	Sumra-1	Dhahian, Sumra-1	40 ± 7.0	40 ± 10	0.49
S. epidermidis	Talah	Talah, Sidr	38 ± 10.9	28 ± 17.9	0.15
Bacillus cereus		Sidr	42 ± 4.4	44 ± 15.1	0.28
Enterobacter aerogenes		Sumra-1	44 ± 5.4	38 ± 4.4	0.04
Serratia marcescens	Talah		40 ± 7.0	48 ± 13	0.13

MIC, minimum inhibitory concentration.

The agar dilution assay technique or a disc impregnated in honey added to the agar inoculated with the microorganism was used for measurement of the antimicrobial activity of honey in the majority of the *in vitro* studies. It was found that a disc impregnated with various concentrations of honey added to an agar plate became dry because of vaporization of fluid from the disc during incubation at 73°C for 24 h (16). In the present study, a series of various concentrations of honey in nutrient broth in which the culture was grown were used. Therefore, it was easy to determine the MIC of honey that inhibited the growth of pathogens. In addition, many studies have used distilled water to obtain various volume/volume concentrations of honey (31−37).

A review of different antibacterial studies showed that the antibacterial properties of honey depend on the geographical origin of the honey (8). In most studies reviewed, the geographical origin of the honey was known, whereas its botanical origin was often not determined. It is highly probable that the antibacterial activity depends mostly on the botanical origin of honey as unifloral honeys from different geographical origins have the same physicochemical properties (38).

A recent review found that darker honeys have a comparable antibacterial activity as manuka honey, honeydew, chestnut, heather and cotton, whereas lighter honeys have a lower antibacterial activity (7). In the present study, potency of amber honey (Talah) is more potent than dark honey (Sumra 1 and 2) although their total antibacterial activity is comparable.

It was assumed that the antibacterial activity of various types of honey are due to sugar, acidity, hydrogen peroxide, methylglyoxal, presence of bacteria in honey, and bee defensin-1. Despite many studies, the exact mechanisms of action are not well identified. Honey may inhibit bacterial growth for a number of different reasons. A recent review showed that antimicrobial activity may be a result of a high sugar concentration, acidity, hydrogen peroxide generation, flavonoids, phenols, or other unidentified components present in the honey (7). Other studies showed that certain honey types contain additional antimicrobial activity including methylglyoxal, defensin-1, and lysozyme (39−43). Some honeys contain bee defensin-1 that are sufficient to inhibit growth of bacteria (20,44,45). However, bee defensin-1 could not be identified in manuka or kanuka honeys (45). Furthermore, it was suggested that the presence of different strains of *L. acidophilus* in honey obtained from different sources may contribute to the differences in the antimicrobial properties of honey (23,24).

We found that the antibacterial activity of honey was stronger in acidic media than in neutral or alkaline media; nevertheless, loss of acidity did not completely abolish its activity (16). Therefore, acidity alone is not the sole agent involved in antimicrobial action. Furthermore, granulated sugar or white sugar applied directly to infected wounds can help to treat infection, and high concentrations were

needed to prevent bacterial growth (46–48). However, honey contains simple sugars and a low concentration of complex sugars; simple sugars were not tested in these reports. Obviously, our previous study demonstrated that simple sugar at concentrations similar to that in honey did not prevent most of the human pathogens tested except for *Klebsiella* spp. and *Proteus* sp., which needed 70–90% sugar concentration for their inhibition (16). Again, the presence of sugar alone may not explain the strong antimicrobial properties of natural honey.

Many studies claimed that the primary antimicrobial component in most honeys is hydrogen peroxide, which is produced by the bee-derived enzyme glucose oxidase (49). There was a high level of correlation between the level of hydrogen peroxide produced by honey samples and their level of antibacterial and antifungal activity (50–52). The antibacterial effect of Slovenian honeys is mostly due to peroxide action (53). It was found that honeys possess DNA degrading activity mediated by honey hydrogen peroxide and an unknown honey component(s) (54). However, it was found that Canola honey and yellow box honey had high hydrogen peroxide levels (754 and 645 μM, respectively) without antibacterial activity or possessing very low antifungal activity, whereas sample R1 had 526 μM hydrogen peroxide but was among the most active of the honeys tested (55). This means that hydrogen peroxide alone may not be an ultimate answer to explain the antibacterial activity of honey. In addition, many studies have found that the level of hydrogen peroxide present in honey is >900-fold lower than expected based on the level of antimicrobial activity; therefore, there may be other natural substances present in honey that optimized the action of hydrogen peroxide (20,56,57). Furthermore, removal of hydrogen peroxide by catalase eliminated bacteriostatic activities caused by both phenolics and hydrogen peroxide. It was concluded that honey phenolics were necessary intermediates that conferred oxidative action of hydrogen peroxide and phenolic/hydrogen peroxide-induced oxidative stress contribute to honey bacteriostatic and DNA damaging activities (54).

Neutralization of peroxide activity with catalase and negligible levels of methylglyoxal present could not inhibit the antibacterial activity of honey (58). Although honey showed synergistic activity when used with antibiotics, methylglyoxal, believed to be the major antibacterial compound in manuka honey, did not act synergistically with rifampicin (59).

In the present study, Sumra-1 and Sumra 2 honeys showed the lowest level of hydrogen peroxide but have good antimicrobial action. This may be attributed to the high content of flavonoids and phenolics. A recent study demonstrated that active honeys possessed significantly higher levels of phenolics of higher radical scavenging activities than honeys of average activity (54). A large body of data has demonstrated the antibacterial action of selected flavonoids (57). Plant-derived flavonoids demonstrated antibacterial, antifungal and antiviral activities (60). The phenolic acid and flavonoid contents of Malaysian Tualang, Gelam, Borneo tropical and manuka honeys exhibited high phenolic contents (15.21 ± 0.51–42.23 ± 0.64 mg/kg), and flavonoid contents (11.52 ± 0.27–25.31 ± 0.37 mg/kg) (61). However, honey samples in the present study contained higher flavonoids and phenolic contents than Tualang, Gelam, Borneo tropical and manuka honeys.

In the present work, Talah honey was more acidic than Sumra 1 and 2 and Sidr honey samples contained high concentrations of sugar and considerable amounts of flavonoids and phenols. This may partially explain its higher potency against bacterial growth. However, it has the lowest action against fungi. Sidr honey has high concentrations of hydrogen peroxides but the lowest potency among the honey samples. This may be due to low levels of flavonoids, phenolic and sugar contents and low acidity. Therefore, none of the identified substances in honey alone can be responsible for its activity. The activity depends on the interaction among the total honey ingredients.

We found that honey increases nitric oxide and stimulates antibody production, which may also explain some of the activities promoted by honey (62–64). Interaction of nitric oxide with other ingredient may play a part in the mechanism of action. This concept needs further experimentation. In general, honey combats bacteria by direct and indirect action. Direct action is based on direct inhibition or killing of bacteria by specific honey components, whereas indirect action of honey induces the antibacterial reaction of the whole body towards bacteria (8).

We may conclude that honey containing more than one active substance has higher potency as an antimicrobial agent. Synergisms among all the contents are essential to table antimicrobial activity. However, further studies are needed to help us understand the mechanism of action and how the various contents of honey synergize its amazing effect.

Antibiotic resistance is a global public health problem and remains a challenging issue (65,66). The U.S. Centers for Disease Control and Prevention (CDC, 2000) has described antibiotic resistance as one of the world's most pressing health problems in the 21st century (67). It is well established that bacterial resistance to antibiotics has increased, and many bacterial infections become resistant to antibiotic treatments. The WHO has identified antibiotic resistance as ''one of the three greatest threats to human health.'' A recent database revealed the existence of >20,000 potential resistance genes (r genes) of nearly 400 different types (68). Antibiotic resistance is increasing while scarce new drugs are being developed to resolve the problem. *S. aureus* and *E. coli* are commonly a cause of human diseases and showed a multiresistant property that has emerged as the major challenging infection (69–73).

The present study adds another scientific clue to the large body of data confirming the ability of various types of honey to inhibit human pathogens whether susceptible or resistant to antibiotics. In addition, the antimicrobial properties of honeys tested in this study do not differ from those of honeys collected from other areas. It is clear that honey poses a considerable antimicrobial activity regardless of the area of origin or plant sources and has a potent activity against multiresistant pathogens. This will pave the way to isolate the most active constituents of honey to be synthesized as medicine for the future.

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